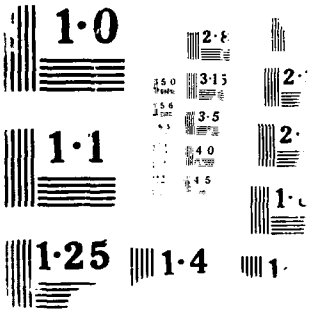
AD-A206 523 DEVELOPMENT OF A DNA-BASED METHOD FOR DISTINGUISHING THE MALARIA VECTORS..(U) EMORY UNIV ATLANTA GA V FINNERTY 15 NOV 87 DAMD17-85-C-5184 1/1

UNCLASSIFIED F/G 6/1 NL

[redacted]

END
DATE FILMED
6 88


1·0
1·1
1·25
1·4
2·8
3·15
3·5
4·0
4·5
2·
2·
1·
1·

AD-A206 523

(2)

AD _____

File copy

Development of a DNA-Based Method for Distinguishing the Malaria Vectors, Anopheles gambiae from Anopheles arabiensis

Annual / Final Report

DTIC
ELECTE
APR 0 6 1989
S D
D

Victoria Finnerty

November 15, 1988

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Ft. Detrick, Frederick, MD 21701-5012

Contract No. DAMD17-85-C-5184

Emory University
Atlanta, GA 30322

Approved for public release; distribution is unlimited.

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

89 [illegible] 330

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT Approved for public release; distribution unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION Emory University 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code) Atlanta, GA 30322	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION US Army Medical Research & Development Comm. 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER DAMD 17-85-C-5184
8c. ADDRESS (City, State, and ZIP Code) Ft. Detrick, Frederick, MD 21701-5102	10. SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO: 62770A PROJECT NO: 3MI-62770A870 TASK NO: AD WORK UNIT ACCESSION NO: 008

11. TITLE (Include Security Classification)
Development of a DNA-based method for distinguishing the malaria vectors Anopheles gambiae from Anopheles arabiensis

12. PERSONAL AUTHOR(S)
Finnerty, Victoria

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual/Final	FROM 85-6-15 TO 87-10-31	1987 November 15	21

16. SUPPLEMENTARY NOTATION
Annual report covers period of time 15 June 1985 - 31 October 1987
Final " " " " 15 June 1985 - 31 October 1987

17. COSATI CODES		
FIELD	GROUP	SUB-GROUP
06	13	
06	01	

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
Recombinant DNA/Species Identification/Anopheline Malaria Vectors, RAI

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

The primary African malaria vectors, A. gambiae and A. arabiensis, belong to a species complex, the members of which are morphologically indistinguishable. Epidemiological studies to determine the involvement of each in malaria transmission were difficult because two or more of the species are commonly sympatric. We have developed a DNA probe (an rDNA fragment from A. gambiae) which reveals RFLPs that distinguish each member of the complex by Southern analysis. The DNA probe method has been extensively tested with both the existing means of distinguishing these species (the isozyme method and the cytogenetic method) and the results in every case were concordant. The probe can sensitively diagnose single adult mosquitoes of either sex, mosquito parts, larval or pupal. Moreover, specimens dessicated in the field and stored up to one year can be scored. The test is compatible with ELISA analyses of dessicated thoraces since the DNA probe can readily diagnose single dessicated abdomens. Blood meal analysis can readily utilize the protein pellet obtained during DNA extraction. We have extended these studies to begin developing a DNA (continued on reverse)

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION	
☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT ☐ DTIC USERS	Unclassified	
22a. NAME OF RESPONSIBLE INDIVIDUAL Mary Francis Bostian	22b. TELEPHONE (Include Area Code) 301-663-7325	22c. OFFICE SYMBOL SGRD-RMI-S

DD Form 1473, JUN 86 Previous editions are obsolete. SECURITY CLASSIFICATION OF THIS PAGE

19. Abstract (continued).
probe method which would utilize a dot blot, and thereby eliminate the need for restriction digests, gel separation, and Southern blotting. Therefore, we have focused our efforts on finding and characterizing species specific sequences within our rDNA clones of A. gambiae and A. arabiensis. Thus far two species specific fragments in A. gambiae have been identified. The characterization of the species specific A. gambiae fragments is expected to provide a focus for future efforts to identify such fragments in A. arabiensis.

FOREWORD

Studies with Recombinant DNA: The investigator has abided by the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules (April 1982) and the Administrative Practices Supplements.

Emory University Biosafety File No. 142-85

Accesion For	
NTIS CRA&I	☑
DTIC TAB	☐
Unannounced	☐
Justification	
By	
Distribution /	
Availability Codes	
Dist	Avail and / or Special
A-1	

TABLE OF CONTENTS

* * *

LIST OF TABLES AND FIGURES

FINAL REPORT

1. Statement of Problem Under Study. Malaria remains the most common disease in the world today, numbering 250-300 million cases at any given time. Among the 92 million new cases each year, there are close to 1 million deaths, nearly all of which are young children. Although malaria is found world-wide, the problem is most acute in subsaharan Africa where the disease presents an enormous obstacle to social and economic development. Although malaria was eradicated from most of its original temperate range in the 1960s, its incidence in the tropics continues to increase, due in part to DDT and chloroquine resistance in mosquito populations. Today, the most common African malaria vectors, Anopheles gambiae and Anopheles arabiensis (1), belong to the Anopheles gambiae species complex, which contains six member species. All of the species are morphologically indistinguishable, and two or three of the species are often sympatric over most of their range. The species differ in behavior and preferred habitat. Moreover, there is evidence suggesting that the two major vector species may not be equally involved in malaria transmission, depending upon the season and location (2). Therefore, one of the requirements for epidemiological studies of these insect vectors is to determine whether an individual female mosquito is infected with the malaria parasite, and also, to what species does she belong. The latter consideration is most pressing for studies of habitat and reproductive behavior which provide information essential for the design of various control strategies. Thus far, the only reliable means of distinguishing among the members of this complex were differences in polytene chromosome banding patterns as observed in either larval salivary gland or adult female ovarian nurse cell tissues (3,4). For field-caught specimens a female would have to be blood fed at least once by the experimenter to insure that ovarian nurse cells could produce the degree of polyteny required for examination. Moreover, interpretation of the chromosomal banding pattern requires considerable skill. Another method, based upon the frequency of certain isozyme patterns (5,6), is not as reliable because even certain diagnostic alleles are found in both species. Moreover, the assay requires that extracts of fresh or frozen specimens be run on polyacrylamide gels. Finally, the A. gambiae and A. arabiensis species can be reliably distinguished by virtue of characteristic HPLC patterns from cuticular hydrocarbons (7,8), but the method is not practical for large numbers of specimens.

2. Background. Many of the major malaria vectors are members of species complexes, for instance, A. culicifacies (9), A. leucosphyrus (10), and the A. farauti sibling series (11). In these complexes, as well as in the A. gambiae complex, reliable species identification of individuals is currently tedious and difficult. Since malaria continues to represent a major world health problem, epidemiological studies with these species is crucial. The proposal hypothesized that the genomic DNA of A. gambiae and A. arabiensis currently differs in ways that would permit reliable species identification. In particular, we sought to develop a species differentiating assay based upon restriction fragment length polymorphism as detected by either heterologous or Anopheles probes.

3. Rationale. Recently, a substantial body of evidence has argued that RFLPs exist between members of closely related species as well as within a single species (12,13,14). We expected to find such differences between A. gambiae and A. arabiensis, and such differences should provide an excellent epidemiological tool. The major advantages of a DNA-based assay are (1) the great sensitivity of Southern analysis so that single mosquitoes (or parts thereof) could be examined, and (2) the ability to use dried material so that field specimens could readily be assayed. In order to find a diagnostic RFLP, we suggested using heterologous

probes such as the highly conserved actin sequence. A second possibility would be to use mosquito mitochondrial DNA or rDNA for RFLP analysis. Since the rDNA proved most useful, its properties are discussed in more detail below.

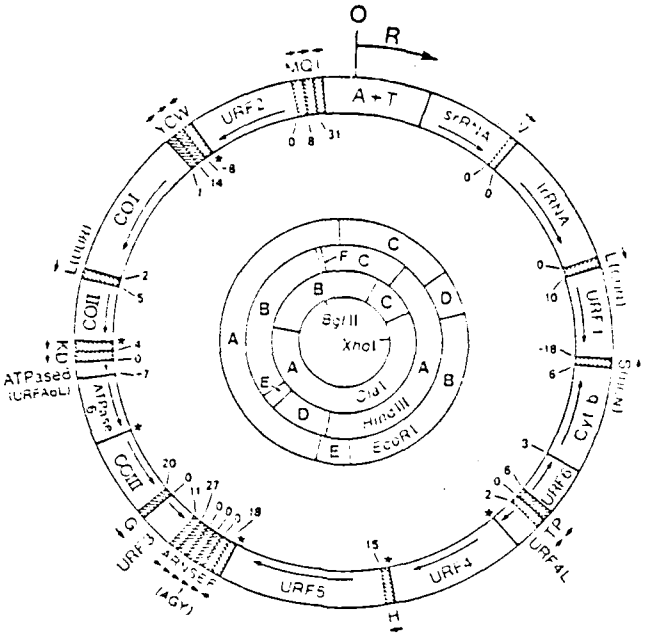
The rDNA genes of most eukaryotic organisms contain spacer regions which, although transcribed, do not appear in the final gene product; these were formerly called NTS (non-transcribed spacer) regions. Such regions have been shown to diverge rapidly compared to the rDNA coding sequences and even single copy genes. Insect rDNA genes also contain intervening sequences (IVS) and/or various other moderately repeated sequences (15). These introns as well as other repeated sequences are similarly much more free to diverge than are coding regions. Quite possibly these IVS could diverge so much that they could lose all homology to those of other members of a species complex.

The rDNA genes have another advantage because thus far, dipterans appear to have these genes present in at least 200 copies per genome arranged in a few large tandem assays (15). Therefore, the rDNA genes possess the ability to yield useful RFLPs as well as species-specific sequences, both of which would be the basis for a diagnostic assay.

4. Experiments and Results. The object of our work was to develop a fast and reliable single mosquito assay to distinguish A. gambiae from A. arabiensis. The first strategy was to determine whether certain heterologous probes would reveal a diagnostic RFLP. The second strategy was to search for diagnostic RFLP by using mosquito mitochondrial DNA. The final strategy was to obtain molecular clones of the rDNA genes of A. gambiae and then to quickly identify non-coding portions of the genes (spacers and IVS).

(A). Utility of heterologous conserved sequences. Our proposal suggested an initial survey of heterologous probes known to be highly conserved throughout various phyla. One choice was the Drosophila actin sequence (16,17) because in addition to its highly conserved sequence, it is a member of a multigene family, thus giving more copies per genome for detection of single mosquito patterns by Southern analysis. However, in our preliminary studies, hybridization with the Drosophila actin probe required such low stringency conditions that the signal to noise ratio made the blots impossible to interpret. We quickly decided to examine appropriate mosquito sequences to use as probes for distinguishing RFLPs. Moreover, since our long term goals include isolation of species-specific mosquito sequences, we did not examine any other heterologous probes.

(B). Mitochondrial DNA probes from A. gambiae. Studies of mitochondrial RFLPs have been used to construct the phylogeny of closely related species. We therefore decided to utilize this approach for A. gambiae and A. arabiensis. We obtained a clone pDyHB from David Wolstenhome, bearing a 4.8kb HindIII fragment from Drosophila yakuba which includes the cytochrome oxidase I and II genes. Since these genes are highly conserved, we sought to use this fragment to identify a homologous sequence in A. gambiae. When pDyHB (shown below in red) was used to probe A. gambiae DNA restricted with various enzyems, Southern analysis showed a small number of hybridizing bands.



Gene map of the 16,019 nucleotide pair Drosophila yakuba mtDNA molecule. The locations of the AeT-rich region, the origin of replication (O), and the direction of replication (R) relative to EcoRI and HindIII restriction sites (inner circles) were determined by electron microscope studies (Fauron and Wolstenholme (1976) Proc. Natl. Acad. Sci 73:3623-3627; (1980) Nucl. Acids Res. 8:2439-2452; Goddard and Wolstenholme (1980) Nucl. Acids Res. 8:741-757). Each tRNA gene (hatched area) is identified by the one letter amino acid code, and individual serine and leucine tRNA genes are identified by the codon family (in parentheses) which their transcription products recognize. Arrows within and outside the molecule indicate the direction of transcription of each gene. The numbers of apparently noncoding nucleotides which occur between the different genes are shown at the gene boundaries on the inner side of the gene map. Negative numbers indicate overlapping nucleotides of adjacent genes. An asterisk indicates an incomplete termination codon (T or TA). The location of all EcoRI, HindIII, ClaI, BglII and XhoI restriction sites, which were used to obtain the clones of segments of the D. yakuba mtDNA molecule from which nucleotide sequences were obtained are shown on the inner map. The letters within the three concentric circles identify the fragments of the restriction enzymes indicated (references attached).

The combined molecular size of these bands was in no case greater than that (14-17kb) expected for the mitochondrial genome. One of the hybridizing bands is a 5.5kb EcoRI fragment which is a convenient size for cloning into the lambda-gt10 vector. We therefore electroeluted 5-6kb DNA from a preparative EcoRI digest, ligated the fragments with lambda-gt10, and packaged the phage using the commercially available "Gigapack." The resulting minilibrary was screened using lowered stringency (hybridization and washing at 42°C) with the pDYHB probe.

One of the selected clones, MR7, had a 5.5kb insert which was used for further study. Of the several enzymes tried, the most interesting differences between gambiae and arabiensis were seen with PstI digests probed with MR7. In addition to the interspecies difference, there was also a suggestion that this probe may be useful for diagnosing various geographical isolates of a given species, such as those listed in table 1. However, we did not investigate this interesting possibility any further, because we questioned whether it actually represents mitochondrial DNA. One possibility would be that it is actually a nuclear DNA se-

quence containing a tRNA also present (see hatched areas above) in mitochondria. Since tRNA sequences tend to be highly conserved, the D. yakuba probe could have selected either nuclear or mitochondrial sequence. We found that a second adjacent fragment, pDvHC (shown in green), also hybridizes to MR7, which indicates that MR7 most likely is a mitochondrial sequence. However, we did not characterize MR7 any further and therefore we cannot make definite statements concerning its origin. Regardless of its origin, MR7 is formally equivalent to a mitochondrial probe since it hybridizes to mitochondrial DNA.

In summary, we found interesting differences between A. gambiae and A. arabiensis using MR7. However, the PstI pattern differences would be difficult to interpret with confidence if one were using single mosquitoes. These differences would be inappropriate in any case for a general diagnostic test. We might have then cloned other portions of the mosquito mitochondrial genome to see if a more useful probe could be found, had we not already obtained interesting preliminary data from mosquito rDNA clones. Thus, we began to examine rDNA probes which might be more useful as potential diagnostic tools.

(C). Isolation of a diagnostic cloned rDNA fragment. An A. gambiae genomic library was screened with a Sciara coprophila rDNA clone (18) which contains one complete cistron. Thirty-two A. gambiae rDNA-containing phage were isolated and selected for further analysis. These clones were restricted with various enzymes and subjected to Southern analysis, in order to find nonconserved regions that might be used to reveal differences between the species. The blots were therefore probed with Sciara rDNA which is not expected to hybridize to fragments from the nonconserved regions. Restriction fragments from such regions (those not hybridizing to the Sciara probe) were then isolated from gels and used to probe genomic Southern blots of A. gambiae and A. arabiensis DNA. Clone λAgr12, shown in figure 1, was found to contain a 0.59kb EcoRI-SalI restriction fragment which consistently showed a different pattern of hybridization to A. gambiae versus A. arabiensis genomic DNA.

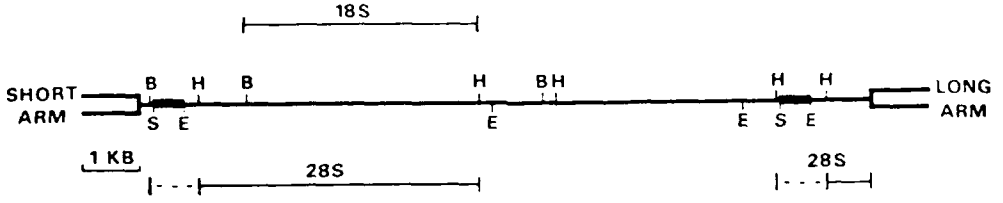


FIGURE 2. λAgr12 restriction map. The approximate locations of the 18S and 28S regions were determined by hybridization with heterologous *Sciara*[14] rDNA (pBC2), and *Calliphora*[18] rDNA (pKB-42 and pKB-33). λAgr12 contains slightly more than 1 rDNA cistron, including the NTS. The dashed line indicates weak hybridization to the heterologous probes. The 0.59 Kb EcoRI-SalI restriction fragment which reveals a diagnostic restriction fragment length polymorphism between *A. gambiae* and *A. arabiensis* is shown as a darkened bar.

The 0.59kb EcoRI-SalI fragment is very close to the 3' terminus of the 28S region of the mosquito rDNA cistron. Hybridization of the Sciara and Calliphora (15) probes is very weak in this region, suggesting a low degree of conservation, yet this fragment is highly conserved among different geographic isolates of the three member species in the A. gambiae complex first examined. EcoRI-SalI genomic digests invariably show the 0.59kb fragment, and there is no evidence for detectable levels of inter-cistronic variation in either of these two restriction sites.

In summary, the probe pAGr12 shows an unambiguous difference between A. gambiae and A. arabiensis as well as A. melas.

(D). The diagnostic fragment is useful for single dried mosquitoes. The 0.59kb fragment was subcloned into the Bluescribe M13+ plasmid (Stratagene Cloning Systems), and this construct, pAGr12A, has been used to probe a large number of A. gambiae complex colonies and field isolates. The A. gambiae complex colonies available are shown in table 1.

Table 1. List of mosquito strains available from the CDC.

Designation	Origin	Species
DMAL	Sudan	A. arabiensis
DONGOLA	Sudan	A. "
KISUMU	Kenya	A. "
G-3	The Gambia	A. gambiae
KWA	Kenya	A. "
BAO	Nigeria	A. "
MU	Tanzania	A. "
ZAN	Zanzibar	A. "
BREF	The Gambia	A. melas

Mosquito genomic DNA was isolated from individual mosquitoes which were simply dessicated at room temperature in the presence of anhydrous calcium sulfate. DNA from individual mosquitoes or mosquito abdomens was isolated by a simple protocol (19), digested with EcoRI, separated on a 0.7% agarose gel, transferred to GeneScreen Plus (NEN Research Products), and then probed with nick-translated pAGr12A. A. gambiae mosquitoes from both east and west Africa show a single, consistent pattern of hybridization to a 1.4kb fragment, and this is illustrated in figure 3a.

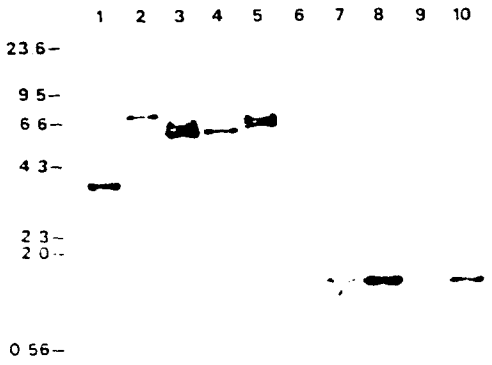


FIGURE 3A Hybridization of pAgr12A to EcoRI digests of single dried female mosquitoes. Species and geographic origin of specimens are as follows: (1) *A. melas* (The Gambia), (2) *A. arabiensis* (Sudan, SENNAR colony), (3) *A. arabiensis* (Sudan, GMAL colony), (4) *A. arabiensis* (Kenya), (5) *A. arabiensis* (Burkina Faso), (6) *A. gambiae* (Tanzania), (7) *A. gambiae* (Zanzibar), (8) *A. gambiae* (Kenya), (9) *A. gambiae* (Nigeria), and (10) *A. gambiae* (The Gambia, G3 colony).

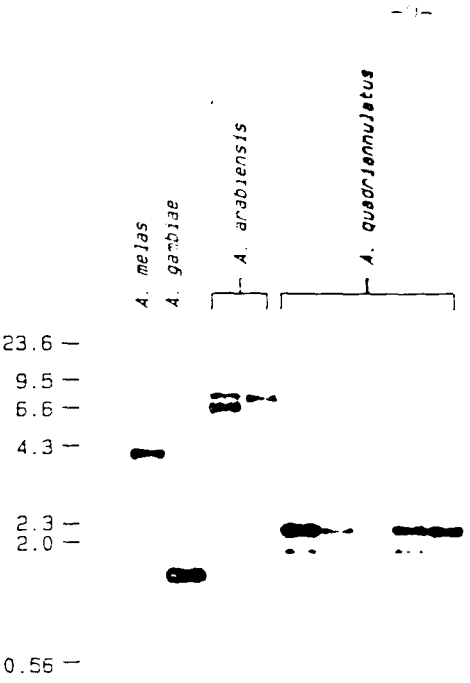


FIGURE 3B. Hybridization of pAGr12A to EcoRI digests of DNA extracted from single female mosquitoes. Species and geographic origin of specimens are as follows: *An. melas*, The Gambia; *An. gambiae*, Zanzibar; *An. arabiensis*, Sudan (GMAL colony and SENNAR colony); and *An. quadriannulatus* mosquitoes from isofemale families.

This fragment corresponds to the 1.4kb segment of AGr12 delimited by the EcoRI site in the probe and the next EcoRI site downstream of the 28S β coding region in the spacer region. None of the more than 1000 individual A. gambiae examined from colonies representing 9 geographically different field isolates revealed any variation in this region. All the A. arabiensis examined to date, representing isolates from four different geographic areas, showed a cluster of EcoRI fragments in the 6-8kb range which contain segments homologous to pAGr12A. We also examined one available A. melas colony which showed a cluster of bands centered around 3.5kb. No individuals from the A. arabiensis and A. melas colonies have been found with the 1.4kb A. gambiae type fragment. Multiple bands of hybridization in A. arabiensis and A. melas are probably due to inter-cistronic variation in the spacer region. In addition, a fourth member of the complex, A. quadriannulatus, is clearly distinguished by the probe, giving a consistent 2.3kb EcoRI band, as illustrated in figure 3b. Therefore, the probe to pAGr12A can be used to examine populations where 2, 3, or 4 of the species are found.

In summary, the diagnostic difference revealed by the probe pAGr12A was found without exception in individual mosquitoes. Further, specimens dessicated by a very simple method show no evidence of DNA degradation even when stored at room temperature for as long as one year. Moreover, in other preliminary experiments we found that other life stages such as second instar larvae and pupae (and obviously both sexes) are readily scored by the DNA probe.

(E). The diagnostic probe is sex linked. Organization of the rDNA cistron appears to be the same in both males and females, as judged by Southern blots of

male and female DNA. However, the intensity of hybridization of pAGri2A to genomic Southern blots, as shown in figure 4, indicates that males have a smaller number of total copies, which is expected if the rDNA genes reside on the X chromosome. A. gambiae-A. arabiensis hybrid female mosquitoes reared in the laboratory contain both of the parental types of rDNA cistrons (Fig. 4). Male hybrids, on the other hand, show the cistron structure of the female parent, indicating that the rRNA genes are located on the X chromosome. This finding directly associates the diagnostic probe with that part of the mosquito genome (the X chromosome) currently used as the basis for cytogenetic speciation.

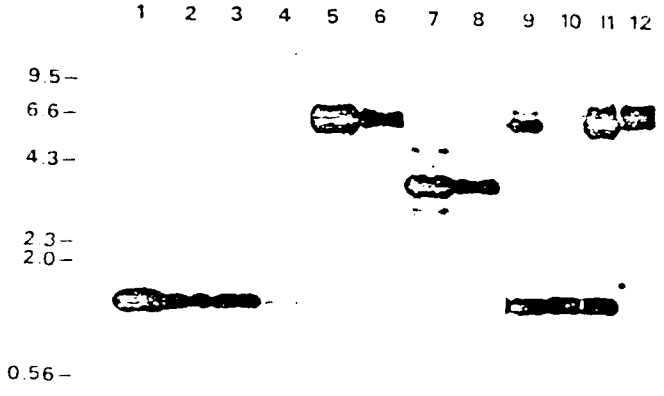


FIGURE 4 Hybridization of pAgri2A to single dried male and female mosquitoes or mosquito abdomens. Lane (1) *A. gambiae* female, (2) *A. gambiae* female (bloodfed), (3) *A. gambiae* female (abdomen only), (4) *A. gambiae* male, (5) *A. arabiensis* female, (6) *A. arabiensis* male, (7) *A. melas* female, (8) *A. melas* male, (9) *A. gambiae-A. arabiensis* hybrid female, (10) *A. gambiae-A. arabiensis* hybrid male, (11) *A. arabiensis-A. gambiae* hybrid female, and (12) *A. arabiensis-A. gambiae* hybrid male. Female parent is listed first for all hybrids. DNA from a single abdomen is clearly more than sufficient to make a species identification. Furthermore, the presence of a bloodmeal in the abdomen does not significantly reduce DNA yield. Dessicated individual pupae and larvae (all instars except the first) can also be readily speciated (data not shown). DNA extraction[19] and hybridization[17] are as described for Fig. 2. Lanes 1–8 were exposed to film, 19 hr; lanes 9–12, 6 hr.

(F). The DNA probe method is compatible with the sporozoite assay and is useful for blood meal analysis. In order to determine whether the probe could be used to assay single mosquitoes which are also assayed for the presence of the malaria parasite, we obtained a number of field specimens which had been dessicated for at least 14 months. The mosquitoes were cut so that Dr. Collins retained the head and thorax for the sporozoite assay (20) and we tested the abdomens. The results, shown in Table 2, indicate that the diagnostic probe can readily distinguish species using only part of a dried specimen.

Table 2. Testing field specimens from *A. gambia* complex mosquitoes collected in Asembo, Kenya, October 1985.

Abdomens from:	Species		DNA not readable
	A. gambia	*A. arabiensis*	
Plasmodium falciparum infected mosquitoes	47 (75%)	17 (27%)	8
Uninfected mosquitoes	78 (49%)	80 (51%)	19

Note: percentages are based on specimens which were identified as to species. The sporozoite assay and DNA probe assay were performed in December 1986.

The proportions of gambiae and arabiensis in Asembo which we found are similar to those found by other workers. Since these specimens were quite old and we did these experiments at a time when our DNA extraction procedure had not been optimized, there are an unacceptable number of unreadable individuals shown in Table 1. Since then, however, we have had few, if any, unreadable abdomens from the specimens so treated.

A second important consideration for a diagnostic probe is whether it is compatible with blood meal analysis. The DNA extraction protocol which we currently use allows blood meal analysis: A single mosquito (or portion thereof) is homogenized in a 1.5ml plastic Eppendorf tube (using a conical glass pestle) with 50µl .08M NaCl, .16M sucrose, .06M EDTA, .5% SDS, .1M Tris-Cl, pH 8.6. The homogenate is incubated, 65°C, 30 min; 8M potassium acetate is added to a final concentration of 1M. After incubation, 4°C, 30 min, and centrifugation at room temperature, 12,000xg, 10 min, supernatant is removed to a fresh tube and the pellet is saved. 100µl 95% ethanol is added to the supernatant and centrifuged again, 12,000xg, 10 min. After discarding the supernatant, the DNA-containing pellet is washed with 70% ethanol, dried and resuspended in 16µl 10mM Tris, 1mM EDTA, pH 8.0. The first (potassium acetate) pellet contains most of the protein. Dr. Collins has examined this protein pellet from a number of the infected specimens listed in Table 1 for blood meal IgG and it can be readily scored for the blood meal source, using commercially available methods (21). Moreover, Dr. Collins has also found that the Plasmodium circumsporozoite antigens can also be detected in this protein pellet with no apparent loss of sensitivity.

(6). The DNA probe method and ODH isozyme method give the same results. The important strategy we employed in comparing the methods was to test isofemale families. For these experiments female mosquitoes collected by Dr. Collins in western Kenya (Ahero, Asembo, and Gombe) and one coastal area (Sabaki) were used. These are heavily infested areas where the two species are known to be sympatric. Ahero is an irrigated, rice-growing area on the Kano plain approximately 10km east of the city of Kisumu. Previous studies of the mosquito fauna of this location have shown that most of the A. gambiae complex mosquitoes breeding in the rice fields are A. arabiensis (22). Asembo is a farming community located on the

north shore of Lake Victoria, approximately 45km west of Kisumu. Gombe is a similar community located at a slightly higher elevation, roughly 20km north of Asembo. The upland Gombe terrain is considerably more hilly and has a longer rainy season than either Asembo or Ahero. Both A. gambiae and A. arabiensis have been reported from these areas (22). Sabaki is a small coastal village approximately 170km north of Mombassa. Using chromosomal methods of species determination, Mosha and Subra (23) reported only A. gambiae and A. arabiensis in this area. All sites fall in the Sudan-Savannah ecological zone. The western Kenya collections were made during mid-May 1986, approximately 6 weeks after the onset of the major rainy season: mosquitoes were collected in Sabaki during mid-August 1986, a considerably drier season. Gravid specimens were placed individually in cotton-covered, 7-dram vials. After oviposition, the vials were capped and shipped back to the CDC insectaries for rearing as isofemale families. The Sabaki larvae were subjected to salinity testing to screen for the presence of the brackish water breeding member of the complex, A. merus, but none were found. Some mosquitoes from each family were analyzed for ODH isozymes (EC 1.1.1.73) by polyacrylamide gel electrophoresis (PAGE) in Dr. Collins' laboratory. Five different ODH alleles were found among the specimens examined: most of the frequently encountered allele combinations are illustrated in figure 5.

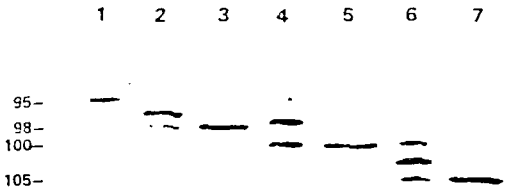


Fig. 5. Octanol dehydrogenase electromorphs found in the Kenya field samples. Lane 1, *A. arabiensis* from the G/MAL colony; lanes 2–3, *A. arabiensis* from Ahero; lane 4, an *A. arabiensis* (G/MAL) × *A. gambiae* (G3) hybrid produced in the laboratory; lanes 5–7, *A. gambiae* from the GO-66 colony established with specimens collected in Gombe.

The number system used to identify the alleles follows Miles (23). The A. gambiae reference colony 16C/SS used by Miles was employed to identify allele 100. Identities of other alleles were inferred from relative mobilities and published allele frequencies. ODH-98, which we found only in specimens from western Kenya, has not previously been described. This number is assigned on the basis of its mobility relative to ODH-95 and ODH-100. The allele was present in 15 of 168 families examined (one family from Asembo was homozygous for the allele). thus it is clearly not rare. Because of the small differences in their relative mobilities, ODH-98 may not have been differentiated from ODH-100 in previously published work that used either starch gel or nonstacking PAGE gel systems.

The most frequent ODH allele in A. gambiae populations is ODH-100, although ODH-105 and ODH-95 have been observed in relatively low frequencies. A. arabiensis populations are typically characterized by the ODH-95 allele, with low frequencies of ODH-90 and ODH-100. Thus, specific ODH alleles do not absolutely identify species (A. gambiae versus A. arabiensis), but genotypes that do not contain the shared alleles can be used to separate the species with a very high degree of reliability.

The DNA probe method of distinguishing species provides a less ambiguous result. All A. gambiae specimens consistently produce the same distinct pattern of hybridization. Figure 6 shows nine individual specimens from the Ahero and Gombe samples tested by the DNA probe; individuals 2, 7, and 8 are scored as A. gambiae, while the other six are A. arabiensis.

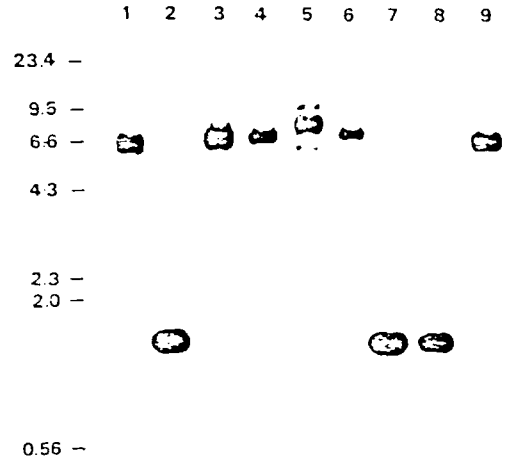


Fig. 6. Hybridization of the pAg112A probe to EcoRI digests field-collected specimens. Lanes 1–5, individual mosquitoes from different Ahero families, lanes 6–9, individuals from Gombe families.

The results for specimens and families analyzed by both DNA probe and ODH isozyme are presented in Table 3. The individual mosquitoes from Sabaki, which were split into two portions and analyzed by both methods, clearly show that the DNA probe and enzymatic methods divide the sample in exactly the same way. All individuals with ODH-100 or ODH-105 alleles test as A. gambiae by DNA probe; those with ODH-95 have the A. arabiensis DNA pattern.

Table 3. DNA-probe and Odh isozyme analyses of *A. gambiae* complex mosquitoes from Kenya

Results for material from Ahero, Asembo and Gombe represent analysis of at least two mosquitoes from each family for DNA type and an additional two mosquitoes for Odh isozymes. Results for material from Sabaki represent DNA-probe and Odh isozyme analyses on single mosquitoes (the abdomen was used for Odh analysis and the head-thorax portion was used for DNA typing)

Location	Probe-checked families		ODH alleles present				
	No.	Probe result	90	95	98	100	105
Ahero	1	*A. gambiae*				+	
	3	*A. arabiensis*		+	+		
	1	*A. arabiensis*		+			
	1	*A. arabiensis*	+	+			
Asembo	1	*A. gambiae*				+	+
	8	*A. gambiae*				+	
	1	*A. gambiae*			+	+	
	1	*A. arabiensis*		+	+	+	
	1	*A. arabiensis*			+		
	1	*A. arabiensis*	+	+	+		
	4	*A. arabiensis*		+	+		
	3	*A. arabiensis*		+			
Gombe	10	*A. gambiae*				+	
	1	*A. arabiensis*		+	+	+	
	1	*A. arabiensis*		+		+	
	1	*A. arabiensis*	+	+		+	
	1	*A. arabiensis*		+	+		
	1	*A. arabiensis*	+	+			
Sabaki	86	*A. gambiae*				+	
	1	*A. gambiae*				+	+
	25	*A. arabiensis*		+			

Specimens from the western Kenya locations were treated so that individuals from each isofemale family were analyzed either by DNA probe (two individuals/family) or ODH isozyme (two or three individuals/family). Of the 41 different families so analyzed, none showed any within-family variation in the DNA probe hybridization pattern. Furthermore, only the expected 1.4kb or 6-8kb bands of hybridization were observed.

All the previously cited studies of ODH alleles in field specimens of A. gambiae and A. arabiensis indicate that, with near certainty, families with only the ODH-100 or ODH-105 alleles can be classified as A. gambiae and families with only the ODH-90 or ODH-95 alleles are A. arabiensis. Indeed, the 20 families from Ahero, Asembo, and Gombe with the A. gambiae isozyme types show the DNA probe pattern diagnostic of A. gambiae. Also, the six families with only alleles ODH-90 or ODH-95 are identified by the probe as A. arabiensis. The fifteen families with other combination of ODH alleles cannot be assigned to species on the basis of their isozyme pattern. However, the DNA probe test of these families indicates that 14 of the 15 are A. arabiensis, a finding that is consistent with those of Miles (24) and others who have reported considerably higher frequencies of ODH isozyme polymorphism in populations of A. arabiensis than A. gambiae. None of the more than 200 individuals examined in this study gave a DNA probe result that would suggest an interspecies hybrid.

In summary, in the 112 individual specimens from Sabaki and the 26 families from the western Kenya sites where isozyme results permit a reliable species diagnosis to be made, the results are in agreement with those provided by the DNA

probe. While this consistent pattern does not represent an absolutely unambiguous validation of the DNA probe method of species determination, it does provide very strong support. This is especially obvious in the mosquitoes from Sabaki, where the two methods subdivide the sample identically.

(H). Comparison of DNA probe and cytogenetic methods for distinguishing A. gambiae from A. arabiensis. Since the chromosome method provides the primary means for unambiguous differentiation of species, Dr. Collins arranged to collaborate with Dr. Vincenzo Petrarca, at the University of Rome, who is expert at the chromosomal diagnosis of these species. Individual gravid females collected in Kenya and Zimbabwe by Dr. Collins were dissected so that ovaries were placed in Carnoy's solution for chromosome squashes. Dr. Petrarca prepared squashes in his laboratory and scored them. The carcasses were dried and brought to Emory for testing with the DNA probe.

The Kenya specimens were collected from Asembo and Ahero, where prior studies (22,23) indicate A. gambiae and A. arabiensis are present. The other specimens were collected from the shore of the Lundi River, from a single site in southern Zimbabwe, where A. arabiensis and A. quadriannulatus have been identified.

The results are shown in Table 4:

Table 4. Results of comparison of DNA and cytogenetic methods of identifying the species of field collected specimens of the Anopheles gambiae complex. All specimens were half-gravid females. Abdomens were preserved in modified Carnoy's fixative for subsequent analysis of ovarian nurse cell polytene chromosomes; head-thorax portions were desiccated in the presence of anhydrous calcium sulfate for later DNA extraction and testing. Only specimens with readable polytene chromosomes were tested by the DNA-probe method.

			DNA PROBE RESULT		
SITE	CYTOGENETIC RESULT	NO.	SAME AS CHROMOSOME	DIFFERENT FROM CHROMOSOME	NOT READABLE
Zimbabwe	An. arabiensis	6	6	0	0
	An. quadriannulatus	55	55	0	0
Kenya	An. arabiensis	108	104	0	4
	An. gambiae	48	44	0	4

All of the specimens from Kenya matched except for two, which were mosquitoes numbered 132 and 133. We believe that these exceptions are due to a mix-up of samples because it is highly unlikely that adjacent tubes would contain the only two exceptions, and these two were therefore dropped from the study. Three of

the chromosomally scored A. gambiae showed bands diagnostic for both species (6-8kb and 1.4kb), but we believe these resulted from partial digestion rather than that they are indicative of hybrids. Approximately 97% of the cytogenetically identified specimens were also identified with the DNA probe. In 8 of the 257 samples, the DNA had been degraded, possibly due to entry of moisture into the microfuge tube containing the carcass. Such degradation is evident in the distribution of DNA fragaments in the agarose gels.

The mosquito chromosome squashes were also categorized as to the type of various polymorphic chromosomal inversions, which are characteristic of each species and are reported to exist in certain frequencies. The data from this aspect of the study showed that there was general agreement between observed and expected frequencies of these rearrangements. These data will be reported in detail elsewhere (see bibliography of publications supported by this contract) and are not treated here.

In summary, 97% of the cytogenetically identified specimens could also be identified with the DNA probe, and in every case the DNA probe and cytogenetic methods of species identification produced concordant results.

(I). Possible use of species-specific rDNA sequences for dot blot assay. As was detailed earlier, a dot blot assay would eliminate the need for running and blotting gels, the use of restriction enzymes, and possibly also obviate the need for a radioactive probe. During the latter portion of the contract period, we explored the possibility that some of the A. gambiae complex rDNA genes could contain species-specific sequences. In particular, certain dipterans are known to have some rDNA genes which contain sequences (IVS) that interrupt the 28S coding region. These IVS should be under fewer selective pressures than other portions of the rDNA genes and could therefore contain sequences which, under normal DNA hybridization conditions, behave in a species-specific manner. We therefore searched for a possible IVS-containing rDNA clone in A. gambiae. Approximately 15 of the previously identified rDNA clones were subjected to Southern analysis and probed with genomic DNA from A. arabiensis. We therefore identified a clone, AGr23, which contained two EcoRI, SalI fragments which were not recognized by the arabiensis DNA probe. This clone (and AGr12) was more carefully mapped and the results are shown in figure 7.

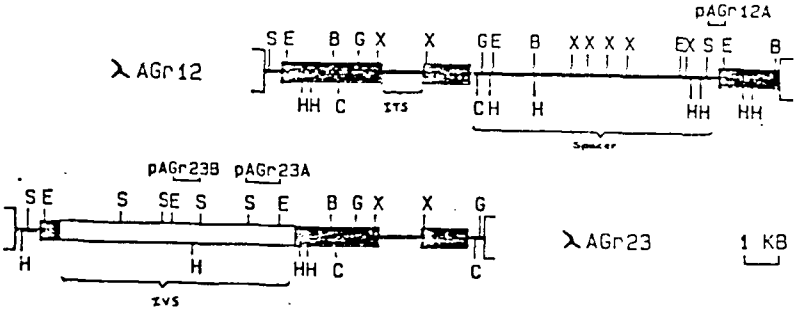


Figure 7. Restriction maps of lambda-Agr12 and lambda-Agr23. Both clones are shown to illustrate the (probable) position of the IVS. As in other dipterans the IVS interrupts the 28S coding sequence. Another feature of the rDNA genes is the internal transcribed spacer (ITS). The species-specific fragments pAGr23A and pAGr23B, as expected, do not contain coding region.

The putative species-specific fragments, shown as pAGr23A and pAGr23B, were subcloned into Bluescript M13 (Stratagene Cloning Systems) and were then used separately to probe Southern blots containing single mosquito digests. All of these mosquitoes were diagnosed as to species using the pAGr12A probe. About 500 individuals were tested in this way, and we found that only the A. gambiae individuals were positive with pAGr23A or pAGr23B; i.e., some gambiae individuals reacted with only one of the two IVS probes. The DNA of A. melas, A. merus, and A. quadriannulatus did not react with either probe. We found that there is individual variation in the intensity of hybridization. This is expected since other dipterans are known to possess variable numbers of such IVS-containing genes (25).

In summary, the two A. gambiae sequences (in pAGr23A and pAGr23B) behave as species-specific probes under the conditions normally used for DNA hybridization. These sequences could be used to design specific probes for dot blot diagnosis of the proportion of A. gambiae individuals in a population. Similarly, these experiments strongly suggest that analogous arabiensis-specific sequences could be obtained, and thereby allow diagnosis of both species from a single dot blot analysis.

Literature Cited

1. Coluzzi M, A Sabatini, V Petrarca, and MA DiDeco. 1979. Chromosomal differentiation and adaptation to human environments in the Anopheles gambiae complex. Trans R Soc Trop Med Hyg 73:483-497.
2. White GB. 1974. Anopheles gambiae complex and disease and transmission in Africa. Trans R Soc Trop Med Hyg 68:278-301.
3. Coluzzi M. 1968. Chromosomi politenici delle cellule nutrici ovariche nel complesso gambiae del genera Anopheles. Parasitol 10:179-183.
4. Coluzzi M and A Sabatini. 1967. Cytogenetic observations on species A and B of the Anopheles gambiae complex. Parasitol 9:73-88.
5. Miles SJ. 1979. A biochemical key to adult members of the Anopheles gambiae group of species. J Med Entomol 15:297-299.
6. Marchand RP and AEP Mnzava. 1985. A field test of a biochemical key to identify members of the A. gambiae group of species in northeast Tanzania. J Trop Med Hyg 88:205-210.
7. Carlson DA and MW Service. 1980. Identification of mosquitoes of Anopheles gambiae species complex A and B by analysis of cuticular components. Science 207: 1089-1091.
8. Hamilton RJ and MW Service. 1983. Value of cuticular and internal hydrocarbons for the identification of larvae of Anopheles gambiae Giles, Anopheles arabiensis Patton and Anopheles melas Theobald. Ann Trop Med Parasitol 77:203-210.
9. Miles SJ. 1981. Unidirectional hybrid sterility from crosses between species A and species B of the taxon Anopheles (Cellia) culicifacies Giles. J Trop Med Hyg 84:13-16.
10. Baimai V, BA Harrison, and L Somchit. 1981. Karyotype differentiation of three anopheline taxa in the Balabacensis complex of Southeast Asia (Diptera: Culicidae). Genetica 57:81-86.
11. Mahon RJ and PM Miethke. 1982. Anopheles farauti No. 3, a hitherto unrecognized biological species of mosquito within the taxon A. farauti Laveran (Diptera: Culicidae). Trans R Soc Trop Med Hyg 76:8-12.
12. Jeffreys AJ. 1981. Recent studies of gene evolution using recombinant DNA. In: Genetic Engineering 2 (R Williamson, ed.), NY, Academic Press, pp. 1-48.
13. Avise J, RA Lansman, and RO Shade. 1979. Use of restriction endonucleases to measure mtDNA sequence relatedness in natural populations. I. Peromyscus. Genetics 92:279-295.
14. Langley CH, E Montgomery, and W Quattlebaum. 1982. Restriction map variation in the AdH region of Drosophila. PNAS/USA 79:5631-5635.
15. Beckingham K. 1980. The ribosomal DNA of Calliphora erythrocephala: An analysis of hybrid plasmids containing ribosomal DNA. J Mol Biol 137:349-373.
16. Ng E and J Abelson. 1980. Isolation and sequence of the gene for actin in S. cerevisiae. PNAS/USA 77:3912-3916.
17. Sanchez F, SL Tobin, U Rdest, E Zulauf, and BJ McCarthy. 1983. Two Drosophila actin genes in detail: Gene structure, protein structure, and transcription during development. J Mol Biol 163: 533-551.
18. Gerbi S and R Renkowitz. 1979. Sciara rDNA has a small homogenous repeat. Mol Gen Genet 173:1-13.
19. Livak K. 1984. Organization and mapping of a sequence on the Drosophila melanogaster X and Y chromosomes that is transcribed during spermatogenesis. Genetics 107:611-634.
20. Collins FH, F Zavala, PM Graves, AH Cochrane, RW Gwadz, J Akoh, and RS Nussenzweig. 1984. First field trial of an immunoradiometric assay for the detection of malaria sporozoites in mosquitoes. Am J Trop Med Hyg 33:227-230.
21. Washino RK and CH Tempelis. 1983. Blood meal analysis. Ann Rev Entomol 28: 179-202.

22. White GB. 1972. The Anopheles gambiae complex and malaria transmission around Kisumu, Kenya. Trans R Soc Trop Med Hyg 66:572-581.
23. Mosha FW and R Subra. 1982. Ecological studies on Anopheles gambiae complex sibling species in Kenya. I. Preliminary observations on their geographical distribution and chromosomal polymorphic inversions. WHO/VBC/82.867.
24. Miles SJ. 1978. Enzymatic variation in the Anopheles gambiae Giles group of species (Diptera: Culicidae). Bull Entomol Res 68:85-96.
25. Gerbi S. 1985. Evolution of ribosomal DNA. In: Molecular Evolutionary Genetics (RJ MacIntyre, ed.), NY, Plenum Press, chapter 7.

Bibliography of Publications Supported by DAMD 17-85-C-5184

Collins FH, AM Mendez, MO Rasmussen, PC Mehaffey, NJ Besansky & V Finnerty. 1987. A ribosomal RNA gene probe differentiates member species of the Anopheles gambiae complex. Amer J Trop Med Hyg 37:37-41.

Collins FH, PC Mehaffey, MO Rasmussen, AD Brandling-Bennett, J Odera, & V Finnerty. 1988. Comparison of DNA probe and isozyme methods for differentiating Anopheles gambiae and Anopheles arabiensis. J Med Entomol 25:116-120.

Collins FH, V Finnerty & V Petrarca. 1988. Ribosomal DNA probes differentiate five cryptic species in the An. gambiae complex. Parasitol (in press).

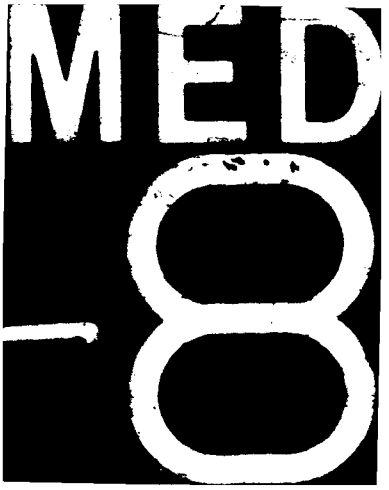
Collins FH, V Petrarca, S Mpofu, AD Brandling-Bennett, JBO Were, MO Rasmussen, & V Finnerty. 1988. Comparison of DNA probe and isozyme methods for differentiating Anopheles gambiae and Anopheles arabiensis. J Med Entomol (in press).

Finnerty V and FH Collins. 1988. Ribosomal DNA probes for identification of member species of the Anopheles gambiae complex. Symp Fla Ent Soc (Daytona Beach, August 1987) (in press).

* * *

DISTRIBUTION LIST

5 copies	Director Walter Reed Army Institute of Research Walter Reed Army Medical Center ATTN: SGRD-UWZ-C Washington, DC 20307-5100
1 Copy	Commander US Army Medical Research and Development Command ATTN: SGRD-RMI-S Fort Detrick, Frederick, Maryland 21701-5012
2 copies	Defense Technical Information Center (DTIC) ATTN: DTIC-DDAC Cameron Station Alexandria, VA 22304-6145
1 copy	Dean School of Medicine Uniformed Services University of the Health Sciences 4301 Jones Bridge Road Bethesda, MD 20814-4799
1 copy	Commandant Academy of Health Sciences, US Army ATTN: AHS-CDM Fort Sam Houston, TX 78234-6100


MED
8